

ASSAYS FOR MODULATORS OF PROLYL-4-HYDROXYLASE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. provisional application Serial No. 60/154,267, filed September 16, 1999. Serial No. 60/154,267 is incorporated by reference as if fully set forth herein.

5 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND OF THE INVENTION

Prolyl-4-hydroxylases (P4H) are enzymes that modify collagen in a
10 manner that stabilizes the conformation of collagen. The synthesis of
hydroxyproline residues by P4H is a critical step in intracellular collagen
processing.

Reduced P4H enzyme activity leads to unstable collagen and disease
symptoms such as those seen in patients suffering from scurvy. Increased
15 activity creates less pliable tissue and is associated with fibrotic diseases.
P4H is recognized as an ideal target for the pharmacological control of
collagen biosynthesis (Bickel, et al., Hepatology August:404-405, 1998).

BRIEF SUMMARY OF THE INVENTION

We have discovered an assay for modulators of P4H enzyme activity
20 in the nematode *Caenorhabditis elegans*. Loss of one isoform of prolyl-4-

hydroxylase causes the nematode to be short and fat, a morphology termed "dumpy" or "dpy". (There are other nematode genes that can be mutated to the dpy phenotype, but there are methods known to one of skill in the art for determining which gene is responsible for the phenotype.) Loss of the
5 second isoform of prolyl-4-hydroxylase while retaining the first isoform of prolyl-4-hydroxylase gives the nematode no apparent phenotype. Mutations in both prolyl-4-hydroxylase isoforms in the same animal result in embryonic lethality. The embryos develop to the pretzel stage and then retract into a mass of cells. These phenotypes provide an easy assay for detecting
10 changes in prolyl-4-hydroxylase activity.

In another embodiment of the present invention, one would introduce the human version of P4H-gene into a P4H-modified nematode and, thus, complement the P4H mutation. One would then expose the test chimeric nematode to a test compound and determine whether the test compound
15 interferes with the P4H activity by examining whether the chimeric nematode or its progeny develop a phenotype that can be attributed to modified P4H activity. We predict that the P4H-modified nematode, which has been exposed to the test compound, will have a phenotype similar to the *dpy-18* mutant or the *phy-1* mutant or the combined *dpy-18; phy-1* double mutant
20 phenotype.

In another embodiment of the present invention, one would attempt to recover P4H activity, thus indicating that the test compound is a P4H activator. In that embodiment, one would introduce a test compound to a

P4H-modified nematode and examine the nematode and its progeny for either recovered P4H activity or a phenotype demonstrating wild-type P4H activity.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

5 Fig. 1 diagrams four different putative prolyl 4-hydroxylase inhibitors.

Fig. 2A and Fig. 2B graphs percent lethality versus concentration of P4H inhibitors. Inhibitor I is depicted in Fig. 2A and Inhibitor II is depicted in Fig. 2B.

DETAILED DESCRIPTION OF THE INVENTION

10 In one embodiment, the present invention is a system designed to look for modifiers (inhibitors and activators) of prolyl 4-hydroxylase activity.

Inhibitors that specifically target human prolyl 4-hydroxylase alpha subunits (I or II) could be used to help people suffering from fibrotic diseases. Activators that specifically target the human prolyl 4-hydroxylase could be
15 used to help treat diseases with Scurvy-like symptoms (underhydroxylated collagen or unstable collagens). Inhibitors or activators that specifically target any protein or molecule with prolyl 4-hydroxylase activity which can function in place of the *dpy-18* gene in the transgenic assay could be used as nematode or drosophila pesticides.

20 In a preferred embodiment, the assay would take place as follows:

Test nematodes will be exposed to a test compound to assay the effect of the compound on prolyl 4-hydroxylase activity. Suitable test nematodes used will include *dpy-18* animal rescued by the human alpha I subunit of prolyl 4-hydroxylase, *dpy-18* animal rescued by the human alpha II subunit of prolyl 4-hydroxylase, wild type *C. elegans*, *dpy-18* mutants, *phy-2* mutants and various *dpy-18;phy-1* mutant combinations. We have included some of these test nematodes to screen for inhibitors of nematode P4H which could potentially be used as pesticides. Combinations of mutant phenotypes could be used to look for specific gene inhibition and potentially specific gene activation. (The Examples below describe the isolation and characterization of the mutants. In general *dpy-18* is a deletion isolated specifically as a knock-out of the P4H gene on chromosome III and PHY-2 is a deletion mutant isolated specifically as a knock-out of the P4H gene on chromosome IV.)

In the methods of the present invention, one may wish to use particular test nematodes with modified P4H activities. Friedman, et al. (Proc. Natl. Acad. Sci. USA 97(9):4736-4741, 2000, incorporated by reference as if fully set forth herein) describes the creation of mutants useful for the present invention. Particularly, Friedman, et al., 2000 describes the creation of *dpy-18* and *phy-2* mutations. In general, we refer to these mutations as "P4H-gene modified nematodes." We refer to the P4H-gene modified nematodes that have been rescued with a human P4H gene as "test chimeric nematodes" or "test chimeric *C. elegans*."

In one embodiment, the test chimeric nematodes or wild-type nematodes will be exposed to test compounds such as chemicals, gene products, and natural products, by various different methods. Preferably, the nematodes will be placed in a solution containing the test compound and
5 soaked for a period of time, or the test compound may be placed directly in the growth medium or on a slide, or introduced through a hole in the egg shell or introduced into the animal by injection into the germline. A suitable length of time would be determined experimentally based on the compound of interest and the age at which one would like to expose the worm.

10 In one embodiment, the test compound is part of a combinatorial chemical library.

If the test compound is an inhibitor of prolyl-4-hydroxylase activity, we expect the nematode's progeny to appear dpy or die, depending on whether the inhibitor is gene-specific or knocks out both prolyl-4-hydroxylase genes.
15 For example, if the inhibitor is gene-specific to the DPY-18 protein, the nematode will appear dpy. If the inhibitor is non-specific and knocks out both P4H genes, the progeny of the tested animal will have a lethal phenotype.

In another embodiment, one would examine the nematodes for the P4H activity level (preferably the P4H:proline ratio). A reduced P4H activity
20 would indicate that the compound is an inhibitor.

In another embodiment of the invention, one could compare the amount of inhibitor needed to affect wild-type, *dpy-18* or *psy-2* mutants. *Dpy-18* and *psy-2* mutations will be more sensitive to inhibitor.

Worms with a *dpy* phenotype appear to be shorter in length (approximately two-thirds wild-type) when viewed with a dissecting microscope. Worms with a lethal phenotype appear to be dead embryos when viewed with a dissecting microscope.

- 5 Activators of prolyl- 4-hydroxylase will rescue the *dpy-18* or *phy-1* phenotype. Potentially, *phy-1* or *dpy-18* nematodes could be exposed to the test compounds and any redundant expression could be activated to rescue the mutant phenotype.

- 10 The Examples below and Friedman, et al., 2000, describe how to create suitable mutants in *C. elegans*. Preferably, the nematode will be one of the genus *Caenorhabditis*, preferably *C. briggsae*. If one wished to use another nematode, such as *C. briggsae*, one of skill in the art would be able to create analogous mutants using the presented information.

EXAMPLES

15 Experimental Procedures

Worm strains

- 20 All wild-type *C. elegans* were from an N2 Bristol strain. Worms were cultured at 20°C under standard conditions unless otherwise noted (J.E. Sulston and J. Hodgkin, Methods. In The Nematode *Caenorhabditis elegans*, pp. 587-606, 1988). LG II:*unc-4(e120)* was used as a marker for transgenic assays. LG III:*dpy-18(ok162)* is a deletion mutation isolated specifically as a knock-out of the prolyl 4-hydroxylase on chromosome III. We found that *dpy-*

18 phenotype corresponds to the absence of prolyl 4-hydroxylase. 11 alleles (mutations in the *dpy-18* gene) are known--*dpy-18*: *e346*, *e364*, *e499*, *e1096*, *e1270*, *e1862*, *h662*, *s361*, *s1304*, *s1305*, *s1306*. LG IV:*unc-22(e66)* is a mutation that can be used to recognize chromosome IV, and *poh-1(ok-177)* is a deletion mutation isolated specifically as a knock-out of the prolyl 4-hydroxylase gene on chromosome IV.

hT2(I:III) is a rearrangement that contains a mutation in the *dpy-18* gene. Thus, *Ht2(I:III)* has a *dpy* phenotype and is not complemented by *dpy-18* mutations.

10 Description of Prolyl 4-hydroxylase Genes in *C. elegans*

Our searches using FASTA and BLAST with the human prolyl 4-hydroxylase sequence against the *C. elegans* genome revealed the presence of two *C. elegans* genes with homology to prolyl 4-hydroxylase. Y47D3B.10 is the transcript which corresponds to the prolyl 4-hydroxylase on LGIII (which we have determined to correspond to the *dpy-18* gene) and F35G2.4 is the transcript which corresponds to the prolyl 4-hydroxylase on LGIV.

Phylogenetic analysis of the two genes compared with that of alpha I and alpha II of human, mouse, rat, chicken, drosophila and a virus prolyl 4-hydroxylase using the programs PILEUP of GCG and PAUP suggest that the two genes are more closely related to each other than to any other sequences.

Isolation of Deletion Mutants

To induce deletion mutations in the two different prolyl 4-hydroxylase genes in *C. elegans* we sent the following primers to Robert Barstead and Gary Moulder at the Oklahoma Medical Research Foundation. These
5 researchers provide a service to the *C. elegans* community by isolating deletions in PCR screens of mutagenized populations. L4 hermaphrodites were treated with trimethylpsoralen and UV light as described (see <http://snmc01.omrf.uokhsc.edu/revgen/RevGen.html> and Dernburg, *et al.*, *Cell* 94(3):387-398, 1998, for a protocol).

10 *mu* 11 > Offspring from mutagenized animals were cultured in groups of 500. After one generation genomic DNA was prepared from pools of worms, and nested primers were used in two successive rounds of PCR. The external primers for Y47D3B.10 (corresponding to *dpy-18*) were CACGACGAGGAAGAGCGACTG and TACGATTTCAGTTCCCAAGC; the
15 internal primers were GAAGAAGCTGTCTGGAGGAGTA and ACGGCTAGTGGGTTGAATCTC. The expected product from amplification of wild-type genomic DNA is 3.2 kb. The external primers for F35G2.4 (corresponding to *poh-1*) were GCTCATGCAGATTTGTTCACT and GTCAGCAGGAAGGCAGTAAAC; the internal primers were
20 GAGCAGAGAAGGATGTAACAA and ATAGTGCGCATTTCGGTTTCA. The expected product from amplification of wild-type genomic DNA is 2.8 kb.

Analysis of Hydroxylated Proline:Proline in Worm Cuticles

As a measure of prolyl 4-hydroxylase activity, the ratio of

4-hydroxyproline:proline was determined in the highly collagenous worm cuticle.

Isolation of Cuticles

To isolate cuticles, worms were bleached and embryos were collected
5 and washed extensively in M9. Embryos were allowed to hatch overnight in M9 and then collected and washed and plated and allowed to grow to L4. L4 worms were collected and washed in M9 and frozen at -80°C . 2ml of packed worms were defrosted and washed with sonication buffer.

Cuticle isolation was performed as a modification of Edgar, et al.,
10 1981. Nematodes were suspended in 3 ml of sonication buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [PMSF], and given ten 20 second bursts of a Branson Sonifier 450 at 50% Duty Cycle and 5-7 output control. Cuticle pieces were collected by centrifugation for 4 minutes at 2000 x rpm in a Sorvall Super T21 and washed several times with
15 10 ml sonication buffer. Cuticles were then transferred to a 1.5 ml microfuge tube, suspended in 1 ml of ST buffer (1% SDS, 0.125 M Tris-HCl, pH 6.8) and heated for 2 minutes in a boiling water bath. The sample was then incubated for 6 hours, spun down for 60 seconds in an Eppendorf microcentrifuge, extracted again with ST buffer as described and left shaking overnight. The
20 disulfide cross-linked proteins of the cuticle were solubilized by heating purified cuticles for 2 minutes in a boiling water bath in 0.5 ml of ST buffer with 5% β -mercaptoethanol (BME). The sample was rocked for 6 hours on a platform shaker and the solution was extracted and the sample was treated

for a second time and left to rock overnight. The insoluble cuticle material was washed several times with distilled water and speed vac dried. All protein samples were stored at -20°C .

Samples for amino acid analysis were hydrolyzed in 6N HCl/0.1% phenol at 110°C for 22 hours and assayed for the ratio of 4-hydroxyproline:proline at MIT's Biopolymer laboratory (Cambridge, MA).

Phenotypes

After receiving deletion mutants in the two prolyl 4-hydroxylase genes we analyzed the phenotypes of the individual mutants and the double mutants.

The fact that the *dpy* phenotype corresponds to the prolyl 4-hydroxylase on LGIII provides an easy method of assaying loss of function of this gene. If one knocks out *dpy-18*, one gets a short, fat, little worm, hence the name "dpy" for dumpy. The *phy-2* gene is wild type at 20°C but is more sensitive to inhibitor concentration than is the wild-type worm, thus allowing one to identify the specific knock-out of this gene.

The double mutant phenotype *dpy-18;phy-2* is an extremely embryonic lethal animals allowing us to look for inhibitors of both genes or all prolyl 4-hydroxylases.

20 RNAi:

Double-stranded RNA was produced using PCR-generated fragments of *phy-1* and *dpy-18* cDNA with T7 promoters linked to primers specific to said DNA. The RNA was then produced using the T7 MegaScript RNA kit

(Ambion). The RNA was injected at 5 mg/ml into N2 animals individually and in combination. The worms were grown at 15 °C, 20 °C and 25 °C. RNA interference technology may be used to create the same knock-out phenotypes as those seen by the deletion mutations.

5 Proposed Isolation of the Human prolyl 4-hydroxylase Alpha I and Alpha II Subunit cDNAs.

Below we describe a proposed method of isolating human P4H gene. One of skill in the art would be aware of modifications and alternative methods that would be equally suitable.

10 The two full-length human prolyl 4-hydroxylase mRNAs have been described in Helaakoski, et al. 1994 (T. Helaakoski, et al., J. Biol. Chem. 269(45):27847-54, 1994) and Annunen, et al., 1997 (P. Annunen, et al., J. Biol. Chem. 272(28):17342-8, 1997.) respectively. Using the sequences described in the above mentioned papers Genbank ACCESSION # M24486,
15 and M24487 corresponding to the two alpha I subunits and ACCESSION # U90441 corresponding to the alpha II subunit one could use the standard BLAST program and search the Genbank database for IMAGE consortium clones.

 If one cannot obtain a full length clone from the IMAGE consortium one
20 could use standard methods such as RT-PCR to create a full-length cDNA from human RNA or a human cDNA library.

Small Molecule Inhibition of Prolyl 4-Hydroxylase Activity.

Small molecules that inhibit protein function can be used to confirm and extend results from genetic experiments. We tested two known prolyl 4-hydroxylase inhibitors for their effects on *C. elegans*. Fig. 1 shows the
5 structures of these inhibitors: 2,4-diethylpyridine dicarboxylate and dimethyloxalyglycine (Inhibitor I and Inhibitor II, respectively). Both inhibitors limit prolyl 4-hydroxylase activity in cells, where their esters are hydrolyzed to form competitors of α -ketoglutarate. We also tested Inhibitor III (which is similar in structure to Inhibitor II) and Inhibitor IV (which is similar in structure
10 to Inhibitor I). Neither Inhibitor III nor Inhibitor II is known to limit prolyl 4-hydroxylase activity in cells.

We exposed adult hermaphrodites that were genotypically wild-type, *dpy-18(ok162)* or *phy-2(ok177)* to varying concentrations of inhibitors. The animal placed in inhibitor was apparently unaffected, but dramatic effects
15 were observed among their progeny. Indeed, when exposed to a high level of Inhibitor I or II (2.7 μ M and 1.3 μ M, respectively), all progeny died, regardless of genotype (Fig 2A and 2B). The dead embryos arrested at the two-fold stage and then exploded; a phenotype reminiscent of the *dpy-18*; *phy-2* dead embryos. This suggests that exposure to the inhibitors results in
20 a lowered prolyl 4-hydroxylase activity.

At a 10-fold lower concentration, the inhibitors affected *dpy-18(ok162)*, but not *phy-2(ok177)* progeny. To ask whether animals with a Dpy phenotype were unusually sensitive to inhibitor, we tested *dpy-10(e128)*, *dpy-11(e224)*,

dpy-13(e184), *dpy-17(e364)* and *dpy-20(e1282)* mutants for inhibitors effects. However, these other *dpy* mutants were comparable to wild-type animals in their response to both inhibitors. Therefore, the sensitivity of *dpy-18* mutants to inhibitors is not caused by its Dpy phenotype. In *dpy-18* mutants, the only
5 prolyl 4-hydroxylase activity remaining is PHY-2, and conversely, in *phy-2* mutants, the only remaining activity is DPY-18. We suggest that the effect of the inhibitor on *dpy-18* mutants reflects inhibition of the remaining PHY-2, and vice versa. Because *dpy-18*, but not *phy-2*, progeny were affected by inhibitor at low concentration, we suggest that PHY-2 is either less abundant
10 or more sensitive than DPY-18.

Both Inhibitor III (at $\leq 29 \mu\text{M}$) and Inhibitor IV ($\leq 3.2 \text{ mM}$) had no effect on the viability of *dpy-18* worms. (See Fig. 1 for structure of Inhibitors III and IV.) These two molecules had not been described previously as inhibitors of P4H.